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**Inhibition of HSV-2 infection by pure compounds from *Thymus capitatus* extract in
vitro**

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ABSTRACT

Thymus capitatus represents one of the five Tunisian species of the genus *Thymus*, which has long standing use for flavouring and preserving several food products. Its constituents have been reported to endow antimicrobial properties, but little is known about their antiviral activities. The aim of this study was to examine the antiviral activity of pure compounds from the most bioactive inhibitory *Thymus capitatus* extract *in vitro* against HSV-2 infection and to identify their mechanism of action. Either the extracts or the essential oil exert inhibitory activity against HSV-2 infection, with the ethanolic extract showing the lowest EC₅₀ value (2.3µg/ml). Three pure compounds were then isolated from the ethanolic extract and investigated for their antiviral activity. β-sitosterol showed the most favorable selectivity index and both cinnamaldehyde and carvacrol exerted moderate antiviral effect. Investigation of the mechanism of action revealed that all three compounds directly inactivated the infectivity of the virus particles. These findings suggest the use of *Thymus capitatus* ethanolic extract as source of anti-HSV-2 pure compounds and warrant further studies to evaluate their therapeutic potential.

Keywords: *Thymus capitatus*, antiviral activity, HSV-2, β-sitosterol, cinnamaldehyde, carvacrol

Chemical compounds: β-sitosterol (PubChemCID: 222284), cinnamaldehyde (PubChemCID: 6428895), carvacrol (PubChemCID: 10364)

Abbreviations: *Thymus capitatus* (*T. capitatus*); herpes simplex virus type 2 (HSV-2); human immunodeficiency virus (HIV); eagle's minimal essential medium (MEM); fetal calf serum (FCS); ethanolic extract (EE); aqueous extracts (AE); essential oil extract (EO); dimethyl sulfoxide (DMSO); milliliter (ml); milligram (mg); grams (g); micrograms (μg); micromolar (μM); [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS); 50% cytotoxic concentrations (CC_{50}); multiplicity of infection (MOI); plaque-forming units (PFU); half maximal effective concentration (EC_{50}); selectivity index (SI); 90% effective concentration (EC_{90}); Nuclear magnetic resonance (NMR); thin-layer chromatography (TLC); deoxyribonucleic acid (DNA)

Introduction

The *Thymus* genus is considered to be one of the largest genera within the *Lamiaceae* family that includes approximately 350 species of perennial, aromatic herb and subshrubs native mainly of Europe, Western Asia and the Mediterranean regions (Stahl-Biskup & Saez, 2002). *Thymus capitatus* (Hoffmanns. & Link, Lamiaceae) represents one of the five Tunisian species of the genus *Thymus*, which has a long standing use for flavouring and preserving several food products. Its essential oil is used in the flavouring cough medicines and oral hygiene products as well as in cosmetic and perfume industry. As a medicinal plant, *T. capitatus* decoction and infusion have traditionally been considered as antispasmodic, carminative, tonic, antiseptic and antitussive drugs (Chiej, 1984). Numerous reports showed antibacterial, antifungal, antioxidant and anti-inflammatory properties of *T. capitatus* extracts and essential oil, rich in carvacrol and thymol (Mkaddem *et al.*, 2010; Achour *et al.*, 2012; Iauk *et al.*, 2014; Maissa *et al.*, 2015). To date, extracts of several species of *Thymus*, as *T. vulgaris* and *T. linearis*, have been investigated for their anti-herpetic effect, even though the constituents responsible of the antiviral activity have not been yet identified (Nolkemper *et al.*, 2006; Schnitzler *et al.*, 2007; Koch *et al.*, 2008; Rajbhandari *et al.*, 2009). Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects about 417 million people aged 15-49 (11%) worldwide, causing genital infections (www.who.int). Most of these infections are asymptomatic but can also cause painful blisters or ulcers at the site of infection. Symptomatic infections are most contagious but sexually transmission can also occur in the absence of symptoms (Roizman, Knipe & Whitley 2007). Of note, genital ulcer disease increases the risk of HIV acquisition since the mucosal damage induces local inflammation, following activation and recruitment of

CD4⁺ HIV target cells (Freeman *et al.*, 2006; Corey, 2007; Feng *et al.*, 2013). Although different anti-herpetic drugs are approved and used to treat acute symptomatic infections (acyclovir, penciclovir and other guanine analogues), the development of new antivirals against HSV-2 is needed due to several drawbacks of available drugs. These include the emergence of drug resistant strains, the inability to eradicate latent infections, the poor availability and the incomplete intestinal absorption (Kimberlin & Whitley, 2007; Cavalli *et al.*, 2012). In this context, alternative natural products from plants have been explored, as sources of antiviral molecules endowed with a mechanism of action different from that of acyclovir (Silva-Mares *et al.*, 2016). Herein, we investigated the antiviral activity of aqueous extract, ethanolic extract and essential oil of *T. capitatus* against HSV-2. β -sitosterol, cinnamaldehyde and carvacrol derived from the ethanolic extract, have been identified as inhibitory compounds against acyclovir sensitive and resistant HSV-2 strains and their mechanism of action has been analyzed.

Materials and methods

Plant identification and collection

Fresh *T. capitatus* (Hoffmanns. & Link, Lamiaceae) plants were collected in May 2014 from Matmata locality in the South East of Tunisia (33° 32' North 9°58' East). Plants was identified by Dr. Marwa Mekni Toujani. Aerial parts of the plants (leaves, stems and flowers) were separated, thoroughly rinsed in running tap water and air dried at room temperature during 14 days. A voucher specimen (TC-001) has been deposited in the herbarium of the Department of Pharmacy of the University "G. d'Annunzio" of Chieti-Pescara.

Chemicals

Eagle's minimal essential medium (MEM) and fetal calf serum (FCS) were purchased from Gibco/BRL (Gaithersburg, MD, USA) and the antibiotic-antimycotic solution (Zell Shield) from Minerva Biolabs GmbH (Berlin, Germany). Acyclovir and heparin were purchased from Sigma-Aldrich (Milan, Italy).

Cells and culture conditions

African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81) were grown as monolayers in MEM supplemented with 10% heat inactivated FCS and 1% Zell Shield.

Virus

A clinical isolate of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV strains were propagated and, when the cytopathic effect involves the whole monolayer, the infected cell suspension is collected and the viral supernatant clarified. The virus stocks were aliquoted, titrated by plaque assay on Vero cells and stored at -80 °C. A HSV-2 strain with phenotypic resistance to acyclovir was generated by serial passage in presence of increasing concentrations of acyclovir, as previously described (Donalisio *et al.*, 2016).

Extracts preparation

The ethanolic and aqueous extracts (EE and AE) were prepared as previously described (Boubaker–Elandalousi *et al.*, 2014). The essential oil (EO) was prepared by dissolving 100 g of dried plant material in 1 liter of distilled water and then submitted to microwave-assisted hydro-distillation at 40 °C during 4 h, in a Clevenger type apparatus. Extracts were kept in a dark flask at 4 °C until tested. Working solutions (25 mg ml⁻¹) of EO and EE were dissolved in dimethyl sulfoxide (DMSO). Aqueous extracts were prepared in a similar way by 24 h maceration.

Isolation of pure compounds from ethanolic extract of *T. capitatus*

Isolation and identification of β -sitosterol. The EE (10 g) of the plant was suspended in *n*-hexane (50 ml) and the resulting mixture was centrifuged at 5000 g for 3 h then filtered. The filtrate was evaporated to dryness under vacuum and the resulting waxy solid dissolved in a 1:1 mixture water: ethanol (50 ml) and extracted with *n*-hexane (5x10 ml) and this latter evaporated to dryness under vacuum. The obtained powder was subjected to purification on a silica gel column chromatography using hexane: dichloromethane 9:1 as the eluent. β -sitosterol (25 mg, purity > 96.3% assayed by HPLC (Figure 1A) following an already reported method (Kakade *et al.*, 2012) was obtained as a white solid and its structure was confirmed by TLC and NMR spectroscopy by comparison with an authentic sample.

Isolation and identification of cinnamaldehyde and carvacrol. The EE (0.5 g) of the plant was subjected to silica gel column chromatography using dichloromethane and 95% dichloromethane / 5% methanol mixture as the eluents. Cinnamaldehyde (10mg, purity > 97.4% assayed by HPLC (Figure 1B)) was eluted first and obtained as yellowish oil and its structure was confirmed by TLC and NMR spectroscopy by comparison with an authentic sample. Carvacrol (12 mg, purity > 96.7% assayed by HPLC (Figure 1C)) was eluted as the second spot and got as a yellowish oil and its structure was confirmed by TLC and NMR spectroscopy by comparison with an authentic sample.

GC and HPLC analysis

GC analysis of the essential oil have been accomplished following the same general procedure as recently reported (Ricci *et al.*, 2017). HPLC analysis were carried out using a Waters 600 HPLC system equipped with a Waters 2996 PDA detector, a

Rheodyne manual syringe-loading valve injector model 7125 (Cotati, CA., USA) fitted with a 20 μ L loop. Data acquisition was monitored by Waters Empower software (ver. 2.0). Chromatographic separation was accomplished employing a GraceSmart RP₁₈ (5 μ m particle size, 250 mm x 4.6 mm, Grace, Deerfield, IL, USA). Column temperature was maintained at 25 \pm 1 $^{\circ}$ C using a cool pocket chiller (ThermoScientific, Waltham, USA). Elution mixture consisted of H₂O and acetonitrile both with 0.1% of formic acid (eluent A and eluent B, respectively). Mobile phase was directly on-line degassed by an Infinity Agilent model 1260 (Agilent Technologies, Santa Clara, CA, USA). The flow rate was 1.20 mL/min. The following gradient elution was used: 15% A – 85% B from 0.01 min. to 13.0 min., 40% A – 60% from 13.01 min. to 16.0 min., 60% A – 40% B from 16.01 min. to 20.0 min., 80% A – 20% B from 20.01 min. to 23.0 min., 90% A – 10% B from 23.01 min. to 26.0 min. The injection volume was 20 μ L. Samples were filtered through a 0.45 μ m membrane polyamide filter before injection.

Method validation was settled according to the “Guidance for Industry-Bioanalytical Method Validation” recommended by Food and Drug Administration (FDA) of the United States. Individual stock solutions for calibration curves of each chemical standard were prepared by dissolution of 10 mg of each reference sample into 10mL of MeOH. The resulting solution was stored in glass-stoppered bottles at 4 $^{\circ}$ C before each HPLC run. Standards for calibration curves and quality control samples (QC), at concentration of 1.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0 and 100.0 μ g/mL, were daily prepared by appropriate dilution aliquots of the stock solutions in MeOH. Pooled quality control samples of analytes were prepared to determine the limit of quantification (LOQ), the intra-and inter-assay precision and accuracy of the method, and to assess the stability of compounds when stored under different conditions. QC

samples at three different concentration levels ($QC_{low}= 5.0$, $QC_{medium} = 45.0$, and $QC_{high}=95.0 \mu\text{g/mL}$) were used to validate or reject the analytical run. On five separate days, six calibration curves were plotted against the corresponding concentrations. Correlation coefficients, slopes, and intercepts of each calibration curve were also evaluated. The limit of detection (LOD), defined as 3 times the standard deviation of a blank samples divided by the analytical sensitivity, was calculated from the calibration graphic following the the guidelines provided by IUPAC s. The LOQ was defined as the lowest concentration on the calibration curve, which could measured ($n=5$) with a precision (RSD %) not exceeding 20% and with an accuracy between 80% and 120% (Fiorito *et al.*, 2017; Taddeo *et al.*, 2017).

Viability assay

Cell viability was measured using the MTS assay as described by Pauwels *et al.* (1988). The effect on cell viability of the different concentrations was expressed as a percentage, by comparing absorbance of treated cells with that of cells incubated with culture medium supplemented with equal volume of DMSO. The 50% cytotoxic concentrations (CC_{50}) and the 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software, San Diego, CA).

Anti-HSV inhibition assay

The effect of *T. capitatus* extracts, isolated compounds or acyclovir on HSV infection was evaluated by plaque reduction assay (Novoa *et al.*, 2016). Vero cells were seeded in 24-well plates at a density of 10×10^4 cells/well. Increasing concentrations of extracts or constituents were added to cells for 2 h; a mixture of different tested substances plus HSV-1 or HSV-2 or acyclovir resistant HSV-2 at a multiplicity of infection (MOI) of 0.001 plaque-forming units (PFU)/cell (100 PFU), were subsequently added to the cells,

which were then incubated at 37 °C for 2 h. The virus inoculum was then removed and the cells washed and overlaid with a medium containing 1.2% methylcellulose (Sigma-Aldrich) and serial dilutions of extracts or constituents. For acyclovir antiviral assay, the compound was added only after infection, diluted in the medium containing methylcellulose as described before. After 24 h (HSV-2) or 48 h (HSV-1) of incubation at 37°C, cells were fixed and stained by using 20% ethanol and 0.1% crystal violet and viral plaques counted. A longer incubation time was used for HSV-1 since this clinical strain generated viral plaques with smaller size than those of HSV-2 at 24 hpi, not suitable for the count. The concentration that reduced the plaque formation by 50% (EC₅₀) was determined by comparing treated and untreated wells using Prism software. Three independent experiments were performed in duplicate.

Virus yield reduction assay

Vero cells seeded in 24-well plates at a density of 10x10⁴ cells/well were pre-treated with serial dilutions of pure compounds for 2 h at 37 °C and infected in duplicate with HSV-2 at a MOI of 0.01 PFU/cell in the presence of the compounds. Following adsorption at 37 °C for 2 h, the virus inocula was removed and cultures were grown in the presence of compounds until control cultures displayed extensive cytopathology. Supernatants were harvested and pooled 48 h after infection and cell-free virus infectivity titers were determined. The aim of the assay was to obtain, if possible, the effective concentration of compound that reduced virus yield by 50% (EC₅₀) as compared to untreated virus controls.

Virus inactivation assay

Approximately, 10⁵ PFU of HSV-2 plus EC₉₀ of pure compounds were added to MEM and mixed in a total volume of 110 µl. The virus-compound mixtures were incubated

for 0 h or 2 h at 37 °C then diluted serially to the non-inhibitory concentration of compounds; the residual viral infectivity was determined by titrating virus by plaque assay on Vero cells, pre-seeded in 96-well plates at a density of 16×10^4 cells/well (Alvarez *et al.*, 2009; Alvarez *et al.*, 2015b).

Pre-treatment assay

Cells were exposed to serial dilutions of pure compounds in a 24-well plate at 37 °C for two hours. After washing, cells were infected with HSV-2 at 0.001 MOI for 2 h, washed and treated as for plaque reduction assay (Donalisio *et al.*, 2016).

Attachment assay

Pre-chilled Vero cells were treated with β -sitosterol or heparin for 30 min at 4 °C and then infected with HSV-2 at 0.004 MOI for 2 h at 4 °C in presence of the pure compound. After three washes with cold MEM to remove unbound virus, cells were overlaid with 1.2% methylcellulose and shifted to 37 °C. After 24 h incubation, cells were stained and viral plaques counted (Alvarez *et al.*, 2009; Alvarez *et al.*, 2015b).

Entry assay

The HSV-2 at 0.004 MOI was adsorbed for 2 h at 4 °C on pre-chilled confluent Vero cells. Cell layers were then washed three times with cold MEM to remove unbound virus, treated with different concentrations of β -sitosterol, and incubated for 3 h at 37 °C. The outer virions were inactivated with acidic glycine for 2 min at room temperature and the cells were washed three times with warm medium and treated as for plaque reduction assay (Alvarez *et al.*, 2009; Alvarez *et al.*, 2015b).

Post-treatment assay

Vero cells monolayers in 24-well plate were infected with HSV-2 at 0.001 MOI for 2 h at 37 °C, followed by two gentle washes to remove unbound virus. Increasing

concentrations of pure compounds were then added to cultures in 1.2% methylcellulose medium. Cells were treated as for plaque reduction assay (Donalisio *et al.*, 2013).

Data analysis

All results are presented as the mean values from three independent experiments performed in duplicate. The EC₅₀ and CC₅₀ values were calculated by regression analysis using the software GraphPad Prism version 4.0 (GraphPad Software, San Diego, California, U.S.A.), by fitting a variable slope-sigmoid dose–response curve. The selectivity index (SI) was calculated by dividing the CC₅₀ by the EC₅₀ value. For virus inactivation and virus yield reduction assays, the viral infectivity in presence and absence of pure compounds was compared using a one-way analysis of variance (ANOVA) followed by Bonferroni test, if P values showed significant differences in virus titers. Significance was set at the 95% level.

Results and Discussion

Inhibitory activity of *T. capitatus* extracts against HSV-1 and HSV-2 infections

Within a project aiming at evaluating the antiviral potential of Tunisian endemic plants, we investigated the antiviral activity of *Thymus capitatus* aqueous (AE) and ethanolic extracts (EE) and essential oil (EO) against HSV-2 infection. To generate dose response curves, assays were performed by treating cells in presence of decreasing concentrations of extracts (ranging from 100 µg/ml to 0.13 µg/ml) before, during, and after viral infection. 24 h post infection, the EC₅₀ was determined by comparing the number of viral plaques in treated and untreated wells, as described in Materials and Methods. As reported in Table 1, the AE, EE and EO exerted an antiviral activity, although to a different extent, with EC₅₀ values of 23.6 µg/ml, 2.3 µg/ml and 18.6 µg/ml, respectively.

In all cases, the antiviral effect was not a consequence of cytotoxicity since the CC₅₀ values on Vero cells were much higher than the EC₅₀ values. Of note, an inhibitory effect of these extracts from *T. capitatus* was also observed against HSV-1, another member of *Alphaherpesvirinae* subfamily, showing EC₅₀ values of 23.4 µg/ml, 16.6 µg/ml and 17.6 µg/ml, respectively. Our data evidenced a minor antiviral activity of EE against HSV-1 rather than that against HSV-2. Although HSV-1 and HSV-2 are structurally and genetically similar, their sensitivity to antiviral compounds may vary depending on the strains and cell type used in antiviral assays as reported for instance in Leary *et al.* (2002). Similar antiviral results were previously observed against Bovine Herpesvirus type-1 infection with EC₅₀ values of 164 µg/ml, 47.8 µg/ml and 3.3 µg/ml, respectively (Boubaker-Elandalousi *et al.*, 2014). These data indicate a broad spectrum of action of *T. capitatus* extracts against Herpes viruses. As reported in Table 1, the EE of *T. capitatus* showed the most favorable selectivity index (SI) value (26.8) against HSV-2 infection and, therefore, it was selected as a source of bioactive compounds.

Chemical characterization of extracts and essential oil

All the obtained phytopreparations (EO, EE, and AE) were screened in order to obtain the respective chemical fingerprint. The essential oil of *T. capitatus* have been analyzed by GC-MS using a well established procedure by our group and employed several times in recent years for the analysis of essential oil (Ricci *et al.*, 2017) (Figure 2).

Inhibitory activity of isolated compounds from *T. capitatus* ethanolic extract and their mechanism of action

Data reported in Table 1 indicate that the EE was the most active one against both viruses under investigation. So we decided to define its phytochemical composition by isolation and structural characterization of its main components. Three compounds were

isolated from *T. capitatus* EE, β -sitosterol, cinnamaldehyde, and carvacrol, and they were tested for their ability to inhibit the replication of the main cause of genital herpes, HSV-2. All of them were active against HSV-2 infection in a dose-response manner, with EC₅₀ values of 2.7 μ M, 39.7 μ M and 51.9 μ M, respectively (Table 2); acyclovir was tested in parallel as a reference drug. Among these compounds, β -sitosterol showed the strongest inhibitory activity with a SI value of 76.2. This is an interesting result since, in a previous study, the same compound isolated from *Euphorbia segetalis* exerted very low HSV-2 plaque reduction (Madureira *et al.*, 2003). Our finding is in agreement with previous data that also showed a strong anti HSV-2 activity for β -sitosterol (Alvarez *et al.*, 2015a). A novel finding is the anti-HSV-2 activity of cinnamaldehyde (SI: 28.3) reported here for the first time. Finally, the anti-HSV-2 activity of carvacrol (SI: 12.1) confirmed previous findings by Pilau *et al.* (2011) and Lai *et al.* (2012). As shown in Figure 2, carvacrol and cinnamaldehyde, were also found as components of the EO, however this was not the most active phytopreparation. Such a discrepancy can be explained by a decrease in bioavailability of both phytochemicals when part of a complex mixture like an essential oil, that in a certain way can modify key parameters relevant to the biological activity like solubility in the medium employed for the antiviral assays. Such an effect has been already described for other biologically active secondary metabolites (Bakkali *et al.*, 2008).

To evaluate whether the antiviral activity of isolated compounds was correlated to virus sensitivity to acyclovir, similar experiments were performed using an acyclovir-resistant HSV-2 (EC₅₀ value of 336.9 μ M for acyclovir). As reported in Table 2, the resistant strain was susceptible to β -sitosterol, cinnamaldehyde and carvacrol, with EC₅₀ values equal to 2.3 μ M, 73.5 μ M and 82.2 μ M, respectively. These data suggest a different

mode of action of the pure compounds from that of acyclovir, a known inhibitor of the HSV-2 DNA polymerase, making these molecules a potential starting point for research and development of new antiviral therapies against HSV-2 infection. Therefore, further experiments were performed to investigate their major mechanism of action.

Firstly, we investigated the ability of pure compounds to directly inactivate HSV-2 viral particles. The assay was performed by mixing a virus aliquot containing 10^5 PFU with a concentration of each compound that reduced almost completely the virus growth ($>EC_{90}$) in the plaque reduction assay. The reduction of the viral titers of treated versus untreated samples was analyzed at high dilutions at which extracts were no more active.

As reported in Figure 3, experiments were performed by incubating the mixtures for 0 h or 2 h at 37 °C. Both β -sitosterol and cinnamaldehyde did not show any significant inhibition when the mixtures were promptly added on the cells without incubation (Figure 3A and 3B). By contrast, after 2 h incubation, both compounds reduced significantly the HSV-2 titer by 99% and 91%, respectively. The ability of β -sitosterol and cinnamaldehyde to directly inactivate HSV-2 infectivity is a novel finding, corroborating the results of Jarikasem *et al.* (2013) who have reported a virucidal activity of a fraction containing β -sitosterol and stigmasterol against both HSV-1 and HSV-2. Furthermore, we have also evidenced the significant ability of carvacrol to inactivate extracellular HSV-2 particles either at 0 h or 2 h of incubation (Figure 3C). This data confirm the results of Jarikasem *et al.* (2013) who have previously shown the virucidal activity of carvacrol against HSV-1 particles. Since the anti-herpetic activity of carvacrol was previously reported in literature, we decided to gain further insights into β -sitosterol and cinnamaldehyde mode of antiviral action. To explore whether they target additional steps of the HSV-2 replicative cycle, specific cell assays we carried

out. As shown in Figure 4A, pretreatment of cells with both β -sitosterol and cinnamaldehyde did not produce any inhibitory effect indicating that upon treatment, the cells remained susceptible to viral infection (pre-treatment assay). This finding exclude that the compounds could act by stably interacting with a cellular component(s) thereby preventing its/their interaction with viral glycoproteins. To evaluate the possibility that the inhibitory activity is due, not only to a virucidal effect, but also to the ability of β -sitosterol to inhibit early steps of the virus replicative cycle, attachment and entry assays were performed. As reported in Figure 4B and 4C, a weak inhibitory activity, around 20-30%, was only observed when the cells were treated with a high dose of compounds (80 μ M) in both assays, whereas a dose response curve was obtained treating cells with heparin, a known inhibitor of attachment. These findings ruled out any effect β -sitosterol and cinnamaldehyde on virus attachment and entry. Then, we wished to investigate whether the compounds were able to block the cell-to-cell transmission of HSV-2 (post-treatment assay). When β -sitosterol and cinnamaldehyde were added to the cells after virus infection they strongly reduced the number of viral plaques as compared to the untreated cells with EC_{50} values of 6.9 μ M and 7.5 μ M, respectively (Figure 4D). This finding indicate their ability to prevent cell-to-cell spread of HSV-2 in a dose-dependent manner at non toxic concentrations. Finally, both β -sitosterol and cinnamaldehyde also reduced the viral titer, in a dose-response manner, when they were tested in a virus yield reduction assay, with EC_{50} value of 3.64 μ M and 8.41 μ M, respectively (Figure 5). These data indicate their ability to inhibit multiple cycles of viral replication at high doses thus limiting the production of viral progenies. Acyclovir was used as standard positive drug of this assay.

Taken together, such antiviral properties might be useful in the setting of a productive infection *in vivo*, where *T. capitatus* extracts or its antiviral compounds might be able to prevent both cell-to-cell spread, which represents a major route of transmission for HSV-2 *in vivo*, and the transmission of extracellular free virus, which is often present in the site of infection at high titers (Dingwell *et al.*, 1994).

In conclusion, this study reports on the anti-herpetic activity of *T. capitatus* extracts and shows, for the first time, the ability of its isolated compounds β -sitosterol, cinnamaldehyde and carvacrol to inhibit infection by acyclovir-responsive and acyclovir-resistant HSV-2 strains. The mechanism of action of pure compounds mainly consists in direct inactivation of HSV-2 extracellular particles along with a reduced cell-to-cell virus spread. Findings identified pure compounds from *Thymus capitatus* ethanolic extract as inhibitors of HSV-2 infection suggesting their potential for treatment of herpetic lesions.

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Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1. HPLC chromatograms of β -sitosterol (A), cinnamaldehyde (B) and carvacrol (C)

Figure 2. GC chromatogram of *T. capitatus* essential oil

Figure 3. Evaluation of virus inactivation by β -sitosterol (panel A), cinnamaldehyde (panel B) and carvacrol (panel C) on infectious HSV-2 particles at 37 °C for 0 h or 2 h. On the y axis, the infectious titers are expressed as plaque-forming units per ml (PFU/ml). Error bars represent standard error of the mean (SEM) of 3 independent experiments. * $p < 0.001$

Figure 4. Effect of β -sitosterol and cinnamaldehyde on viral replicative cycle. Pre-treatment assay (A), attachment assay (B), entry assay (C) and post-treatment assay (D). Heparin was used as a known inhibitor of attachment. Acyclovir were used as standard positive drugs when it was added after infection. Error bars represent the SD of the mean of three independent experiments.

Figure 5. Effect of β -sitosterol (panel A), cinnamaldehyde (panel B) or acyclovir (panel C) on multiple cycles of HSV-2 replication. Viral titers (expressed as PFU/ml) are shown as means plus standard error of the mean (SEM) for three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 1. Antiviral activity of *Thymus capitatus* extracts

	Virus	EC50^a (µg/ml) – 95% C.I. ^b	CC50^c (µg/ml)	SI^d
Aqueous extract	HSV-2	23.6 (15.5 - 30.2)	> 300	> 12.6
	HSV-1	23.4 (14.3 - 32.4)	> 300	> 12.7
Ethanollic extract	HSV-2	2.3 (1.4 - 3.8)	62.5	26.8
	HSV-1	16.6 (11.2 - 24.6)	58.5	3.5
Essential oil	HSV-2	18.6 (13.4 - 25.9)	129.1	6.9
	HSV-1	17.6 (6.5 - 27.5)	107.0	6.0

^a half maximal effective concentration^b confidence interval^c half maximal cytotoxic concentration^d selectivity index

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Table 2. Antiviral activity of purified compounds derived from *Thymus capitatus* ethanollic extract

	Virus	EC50^a (µg/ml) – 95% C.I. ^b	CC50^c (µg/ml)	SI^d
β-sitosterol	HSV-2	2.7 (1.8 - 4.1)	212.8	76.2
	HSV-2 ACV-r ^e	2.3 (2.1- 2.5)	212.8	90.9
Cinnamaldehyde	HSV-2	39.7 (30.4 - 51.8)	1126	28.3
	HSV-2 ACV-r	73.5 (47.3- 94.3)	1126	15.3
Carvacrol	HSV-2	51.9 (36.3-74.2)	632.9	12.1
	HSV-2 ACV-r	82.2 (62.3-108.5)	632.9	7.6
Acyclovir	HSV-2	0.7 (0.4-1.0)	754	1062
	HSV-2 ACV-r	336.9 (263.3-395.3)	754	2.2

^a half maximal effective concentration^b confidence interval^c half maximal cytotoxic concentration^d selectivity index^e HSV-2 acyclovir resistant strain

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